Allelotyping in Mycosis Fungoides and Sézary Syndrome: Common Regions of Allelic Loss Identified on 9p, 10q, and 17p

Julia J. Scarisbrick, Alison J. Woolford, Robin Russell-Jones, and Sean J. Whittaker

Skin Tumour Unit, St John's Institute Dermatology, St Thomas' Hospital, London, U.K.

Allelotyping studies have been extensively used in a wide variety of malignancies to define chromosomal regions of allelic loss and sites of putative tumor suppressor genes; however, until now this technique has not been used in cutaneous lymphoma. We have analyzed 51 samples from patients with mycosis fungoides and 15 with Sézary syndrome using methods to detect loss of heterozygosity. Microsatellite markers were selected on 15 chromosomal arms because of their proximity to either known tumor suppressor genes or chromosomal abnormalities identified in previous cytogenetic studies in cutaneous lymphoma. Allelic loss was present in 45% of patients with mycosis fungoides and 67% with Sézary syndrome. Loss of heterozygosity was found in over 10% of patients with mycosis fungoides on 9p, 10q, 1p, and 17p and was present in 37% with early stage (T_1 and T_2) and 57% with advanced disease (T_3 and T_4). Allelic loss on 1p and 9p were found in all stages of mycosis fungoides, whereas losses on 17p and 10q were limited to advanced disease. In Sézary syndrome high rates of loss of heterozygosity were detected on 9p (46%) and 17p (42%) with lower rates on 2p (12%), 6q (7%), and 10q (12%). There was no significant difference in the age at diagnosis or number of treatments received by those with loss of heterozygosity and those without, suggesting that increasing age and multiple treatments do not predispose to allelic loss. These results provide the basis for further studies defining more accurately chromosomal regions of deletions and candidate tumor suppressor genes involved in mycosis fungoides and Sézary syndrome. ***Key words: cutaneous T cell lymphoma/loss of heterozygosity/tumor suppressor gene. J Invest Dermatol 117:663–670, 2001***

Mycosis fungoides is the most common form of primary cutaneous T cell lymphoma (CTCL) with an incidence of 0.36 per 10^5 person-years (Weinstock and Gardstein, 1999). It is generally a low-grade lymphoma, which typically presents with cutaneous patches and plaques; however some patients develop progressive disease with cutaneous tumors, erythroderma, or visceral spread. Sézary syndrome is a leukemic form of CTCL with an aggressive clinical course; it is rarer than mycosis fungoides accounting for 2–10% of new cases of CTCL (Willemze *et al*, 1997; Zackheim *et al*, 1999). It typically presents with lymphomatous involvement of more than 90% of the skin (erythroderma), intense pruritus, peripheral lymphadenopathy, atypical lymphocytes with convoluted nuclei in the peripheral circulation (Sézary cells), and a peripheral blood T cell clone as demonstrated by T cell receptor (TCR) gene analysis studies (Russell-Jones and Whittaker, 1999).

Little is known about the molecular pathogenesis of mycosis fungoides and unlike nodal lymphomas specific chromosomal abnormalities have not been identified. Cytogenetic studies in nodal lymphomas and leukemia have been very rewarding and have identified several disease-specific abnormalities (Weiss *et al*, 1987; Kurzrock *et al*, 1988; Chiou *et al*, 1994; Argatoff *et al*, 1997; Beylot-Barry *et al*, 1998). Attempts to identify these well-defined chromosomal abnormalities in primary cutaneous lymphoma have not been fruitful. The t(14; 18) that characterizes nodal follicular center cell lymphoma was found in only one of 14 cases of primary cutaneous follicular center cell (Cerroni *et al*, 1994). Similarly, the t(2;5) characteristic of nodal $CD30^+$ large cell anaplastic lymphoma was not found in 14 cases of primary cutaneous $CD30^+$ large cell anaplastic lymphoma (DeCoteau *et al*, 1996). These findings suggest that primary cutaneous lymphomas have a different pathogenesis from their nodal counterparts despite similar morphologic features.

Peripheral blood cytogenetic studies in CTCL have been ongoing since 1968 (Spiers *et al*, 1968). Clonal chromosomal abnormalities have been identified in 20–100% of patients with Sézary syndrome and in the peripheral blood of some patients with advanced stages of mycosis fungoides. Several cytogenetic studies in patients with advanced CTCL have identified multiple clonal chromosomal abnormalities, most commonly involving structural rearrangements of chromosomes 1, 2, 6, 9, 14, and 16 and numerical abnormalities of 6, 8, 10, 11, 13, 17, and 21.

A recent study of 11 patients with CTCL identified chromosomal loss between 1p22 and 1p36 in five patients and rearrangements of chromosome 2 in four patients (Thangavelu *et al*, 1997). Rearrangements involving 2p were also detected in two of six patients by Berger *et al* (1988) who reviewed the literature to identify two common breakpoints on 2p; one clustering around 2p11–14 and the other around 2p23–25. Complete or partial monosomy of chromosomes 8p, 17p, and isochromosome 17q have

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Reprint requests to: Dr. Julia Scarisbrick, Skin Tumour Unit, St John's Institute Dermatology, St Thomas' Hospital, Lambeth Palace Rd., London, SE1 7EH, U.K. Email: juliascarisbrick@doctors.org.uk

been identified in CTCL (Nowell *et al*, 1982; Berger *et al*, 1988; Thangavelu *et al*, 1997). Deletions of 6q have also been identified in several studies (Thangavelu *et al*, 1997; Nowell *et al*, 1982; Shapiro *et al*, 1987). Rearrangements involving 9p were identified in three of six patients with CTCL by Berger *et al* (1988) in addition one patient had a rearrangement involving 14q32. This locus was also involved in a translocation in two of 23 patients with CTCL in a previous study by Nowell *et al* (1986). Studies of Sézary syndrome have identified frequent abnormalities also involving 1p, 2p, 6q, 9, 10q, 13q, 17p, and 21 (Johnson *et al*, 1985; Sole *et al*, 1988; Limon *et al*, 1995; Brito-Babpulle *et al*, 1997).

Most cytogenetic studies have been based on analysis of metaphases from peripheral blood lymphocyte cultures even though morphologic abnormalities are seldom present in the peripheral blood of patients with MF. These multiple chromosomal abnormalities have mainly been found in patients with advanced stages of mycosis fungoides and are associated with a poor prognosis (Thangavelu *et al*, 1997; Whang-Peng *et al*, 1979; Shapiro *et al*, 1987). Studies of skin biopsy material from patients with mycosis fungoides have generally not been successful for a variety of biologic and technical reasons. The growth of tumor cells from solid tissues in culture is often poor because of a low mitotic index. Cultures may be contaminated with keratinocytes, fibroblasts, or reactive lymphocytes and preparations of good quality metaphases from solid tumor can be difficult.

Although rearrangements of specific oncogenes have rarely been found in CTCL, studies of tumor suppressor genes have been more rewarding. Inactivation of P53 has been found in advanced mycosis fungoides and Sézary syndrome (McGregor *et al*, 1995, 1999; Lauritzen *et al*, 1995; Marks *et al*, 1996; Marrogi *et al*, 1999), which is similar to findings in nodal non-Hodgkin's lymphoma and suggests that P53 gene mutations are associated with disease progression in lymphoma (Gaidano *et al*, 1991). Abnormalities of the P16 gene were detected in seven of nine patients with mycosis fungoides in both early and advanced stages of disease (Navas *et al* 2000). In addition a further study found absent expression of the P16 gene in five of 20 patients with early stages of mycosis fungoides (Peris *et al*, 1999). Germline mutations of P15 and P16 genes are found in some cases of familial melanoma (Hussussian *et al*, 1994) and inactivation of these genes has also been found in non-Hodgkin's lymphoma (Gombart *et al*, 1995; Heyman *et al*, 1996).

We have performed an extensive allelotyping in order to identify patterns of loss of heterozygosity (LOH) in both early and advanced cutaneous stages of mycosis fungoides and Sézary syndrome. Allelic loss is a common mechanism of tumor suppressor gene inactivation and provides information on the likely involvement of such genes in the pathogenesis of mycosis fungoides and Sézary syndrome.

MATERIALS AND METHODS

Sample selection Tumour DNA from lesional skin and normal control DNA from the patients' peripheral blood mononuclear cells or lymph nodes, was extracted according to standard procedures by proteinase K digestion and phenol/chloroform extraction, from individual patients with mycosis fungoides. All tumor samples were chosen because, either southern blot or single strand conformational polymorphism–polymerase chain reaction (PCR) based TCR gene analysis (Whittaker *et al*, 1991; Fraser-Andrews *et al*, 2000) had previously demonstrated a clonal population in lesional skin, which was absent from the peripheral blood or lymph node. Furthermore, only those samples with a large dominant clone were selected for this study: a large dominant clone was determined by measuring the signal intensity of the discrete band, using Image Master Scanning Densitometer (Amersham Pharmacia, Piscataway, NJ) with Image Master Softwear (Amersham Pharmacia) and only samples with a signal intensity of more than 50% in the discrete band compared with the background smear were considered to contain a large dominant clone.

These samples consisted of 51 patients with mycosis fungoides with different cutaneous stages of disease: $T_1 = 14$, $T_2 = 16$, $T_3 = 17$, and $T_4 = 4$, which included IA = 14, IB = 13, IIA = 3, IIB = 11, III = 1, and IVA = 9 (Lamberg *et al*, 1984) and 15 patients with Sézary syndrome (III = 8 and IVA = 7).

Microsatellite analysis Microsatellite analysis requires comparison between tumor and normal DNA from individual patients. In most patients with mycosis fungoides tumor DNA was extracted from lesional skin and normal control DNA from either peripheral blood lymphocytes or lymph nodes. All 15 cases with Sézary syndrome had a large dominant T cell clone in peripheral blood. In three cases DNA from lymph nodes did not demonstrate a T cell clone and these samples were used as a normal control. In the other 12 cases with Sézary syndrome and three patients with tumor stage mycosis fungoides T cell clones were detected in skin, blood, lymph nodes, and bone marrow aspirates. In these patients no DNA was available without a T cell clone and normal DNA was obtained by isolating $CD8^+$ cells from peripheral blood lymphocytes as described below.

Prior to isolation of $CD8^+$ cells, formalin-fixed paraffin-embedded histologic sections from lesional skin were immunostained using CD4 and CD8 monoclonal antibodies (Vector Laboratories, Burlingame, CA) to confirm that the tumor cells had a typical $CD4^+$ $CD8^-$ phenotype (Willemze *et al*, 1997).

First, lymphocytes were separated from whole blood using Lymphoprep (N Fiemed, Norway) and centrifuged at 1400 r.p.m. for 25 min with gentle acceleration and deceleration. The lymphocytes were then removed and washed using phosphate-buffered saline (Sigma, Poole, U.K.) with centrifugation at 1400 r.p.m. for a further 10 min. The lymphocyte pellet was then resuspended in phosphate-buffered saline with 2% fetal bovine serum (Helena Technologies, Barnsley, U.K.). Viable lymphocytes were counted using a Neubaeu hemocytometer with trypan blue dye exclusion. This manual counting method includes all lymphocytes (both T cells ($CD3^+$, including $CD4^+$ and $CD8^+$ lymphocytes) and B cells ($CD20^+$). A more accurate estimate of the number of $CD8^+$ lymphocytes was then calculated using the percentage CD3 count and CD4/8 ratio for each sample. Cells were stored in liquid nitrogen for future isolation of the $CD8^+$ lymphocytes. CD8 Dynabeads (Dynal AS, Norway) were used to isolate $CD8^+$ cells following the manufacturers' instructions. DNA extraction was then performed using Genomic Prep Cells and Tissues DNA isolation kit (Amersham Pharmacia) in accordance with manufacturers' instructions. DNA was precipitated by ethanol and resuspended in 50 μl of water.

Tumor and normal DNA samples from patients with mycosis fungoides were analyzed for allelic loss using oligonucleotide primers for 25 microsatellite markers from 15 chromosomal arms. In Sézary syndrome 17 microsatellite markers on eight chromosomal arms were analyzed. This more limited analysis was performed due to the limited amount of normal DNA available for comparison in LOH studies.

Microsatellite markers were chosen with high rates of heterozygosity (> 0.8) and were either close to chromosomal regions found to be deleted in previous cytogenetic studies of CTCL or known tumor suppressor genes (**Table I**). These included 21 dinucleotide markers: Primer sequences were taken from the Genethon Human Linkage Map (Gyapay *et al*, 1994), excluding U09579, RH70320, L11910 (EMBL, Meyerhofstr, Germany), IFNA, p15, p16 (Heyman *et al*, 1996), p53 (Futreal *et al*, 1991), and four mononucleotide repeats: BAT 26, BAT RII, BAT 34C4 (Zhou *et al*, 1997), and the G_8 tract of the BAX gene (Molenaar *et al*, 1998).

Primers for microsatellite markers were amplified using the PCR in a reaction mixture of 20 μl, including 20 pmol of each oligonucleotide primer, 0.2 mM deoxyadenosine triphosphate, 0.2 mM deoxyguanosine triphosphate, 0.2 mM deoxythymidine triphosphate (Amersham Pharmacia), 0.03 mM deoxycytidine triphosphate, 0.1 μCi [α-^{33}P]-deoxycytidine triphosphate (3000 Ci per mmol), 50 ng of sample DNA, 1 × PCR buffer, containing 1.5 mM $MgCl_2$, 1.25 units Taq polymerase (Amersham Pharmacia) and 1.25 units Taq antibody (Clontech, Basingstoke, U.K.). Prior to PCR amplification the mixtures were denatured at 94°C for 5 min. Twenty-five to 30 cycles of PCR were performed in a DNA Perkin Elmer, Wellesley, MA 9700 thermal cycler. Each cycle consisted of denaturing at 94°C for 20 s, annealing at 55–65°C (according to the T_m for each oligonucleotide primer pairs) for 20 s and extension at 72°C for 50 s with a prolonged extension of 5 min during the final cycle.

A negative control reaction containing no DNA was examined for each PCR assay. After agarose gel electrophoresis, to assess efficiency of amplification, PCR products were diluted 2-fold with stop solution (95% formamide, 20 mM ethylenediamine tetraacetic acid, 0.05% xylene cyanol FF, and 0.05% bromophenol blue) and denatured for 10 min at 95°C before being loaded on to denaturing 6% polyacrylamide (Gibco BRL, Gaithersburg, MO) gels containing 7 M urea (United States-Biochemicals, Swampstott, MA) and electrophoresed using a S2 sequencing gel apparatus (Gibco BRL). The gel was dried on 3 mm Whatmann

Table I. Summary of chromosomal locations analyzed with reference to previous cytogenetic findings and candidate tumor suppressor genes

Chromosomal region	Regions of allelic loss in CTCL	Candidate TSG	Microsatellite markers
1p22–36	1[1,2], 1p22–36[3]	P73[16], P18[17], TAL-1[18]	D1S201, D1S247
2p16–21	2p14–25[1], 2p11[4], 2p21[5]	hMSH2,hMSH6[19,20]	BAT26, D2S123
3p22		TGF-beta receptor 2[21]	BAT-RII
5q13,21–22,5q35		P21[22],APC[23]	U09579, D5S346, D5S677
6q16.3–21,21–23.3	6q[3–8], 6q21–23[9]		D6S283, D6S261
9p21	9[4,8], 9p[1]	P15,P16[24,25]	IFNA, p15,16
10q23–24	10[3,5,8], 10q22–26[2,10–12]	PTEN[26], FAS[27]	D10S215, D10S541, D10S209
11q23	11[1,10]	ATM[47]	D11S1347
12p12.3	12[10,13], 12p11–13[2]	LRP6[29]	T68115
13q14.2–22	13q[5,8],13q21–22[10,11]	Rb[30]	L11910, D13S160
14q32	14q[13], 14q32[1,14]		D14S1054
17q11.2–12	Iso17q[4,7,9]	BRCA1[31], NF1[32]	D17S250
17p13.1	17p[2,3], 17p13[15]	P53[33–36]	BAT34C, p53
19q		BAX[37]	BAX(G_8tract)
21q22.1	21[4,10]	TIAM-1[38]	D21S263

[1]Berger *et al* (1988). [2]Limon *et al* (1995). [3]Thangavelu *et al* (1997). [4]Van Vloten *et al* (1980). [5]Sole *et al* (1988). [6]Shapiro *et al* (1987). [7]Nowell *et al* (1982). [8]Whang-Peng *et al* (1982). [9]Johnson *et al* (1985). [10]Edelson *et al* (1979). [11]Karenko *et al* (1997). [12]Karenko *et al* (1999). [13]Barbieri *et al* (1986). [14]Nowell *et al* (1986). [15]Brito-Babpulle *et al* (1997). [16]Kawano *et al* (1999). [17]Hatta *et al* (1997). [18]Janssen *et al* (1993). [19]Lowsky *et al* (1997). [20]Indraccolo *et al* (1999). [21]Lagneaux *et al* (1997). [22]Villuendas *et al* (1997). [23]Baeg *et al* (1995). [24]Gombart *et al* (1995). [25]Heyman *et al* (1996). [26]Nakahara *et al* (1998). [27]Komada *et al* (1997). [28]Takeuchi *et al* (1998). [29]Baens *et al* (1999). [30]Pescarmona *et al* (1999) [31]Beckmann *et al* (1996). [32]Bollag *et al* (1996). [33]McGregor *et al* (1995). [34]Lauritzen *et al* (1995). [35]McGregor *et al* (1999). [36]Marks *et al* (1996). [37]Molenaar *et al* (1998). [38]Chen *et al* (1995).

paper and exposed to X-ray film (Genetic Research Instrumentation, Mansfield, WI) with an intensifying screen (Appligene Oncor, Illkirch, France) at room temperature for 48–72 h.

Determination of LOH All samples where two distinct allelic bands were present in the normal DNA were considered to be informative. Samples that produced a single allelic band in normal DNA or failed to amplify for a given microsatellite marker were scored as noninformative.

The signal intensities for all informative samples were examined visually by two independent observers without knowledge of clinical details. LOH was scored as positive when a clear reduction in signal intensity was detected in one of the alleles of the tumor DNA compared with the same allele in the paired normal DNA.

Microsatellite analysis using different markers produces different allelic banding patterns and LOH needs to be assessed in comparison with other samples using the same marker. The number of bands representing one allele varies markedly for different markers but is relatively constant for each marker using different patient samples (**Fig 1**). Multiple allelic bands occur due to slippage of DNA polymerase on the template sequence, which may be marked because of the repetitive nature of the microsatellite sequence (Newton and Graham, 1997). We attempted to reduce this slippage by limiting the number of PCR cycles to 25 and lowering the primer concentration. In addition a higher number of bands are more frequently seen using ^{33}P compared with ^{32}P because a sharper and finer banding pattern is produced (Payne, 1997). Finally, the intensity of the upper group of bands, representing the larger allele, was often less due to preferential PCR amplification of smaller sized products (Payne, 1997).

In instances where more than one band was present within each allele the exact position of each allele was decided by comparing the banding pattern in all samples analyzed with the same marker and selecting the most consistent pattern: the two alleles were identified as two groups of bands of similar number and similar signal intensity (**Fig 1**). In order to quantify signal intensities we also analyzed samples using a scanning densitometer with Image Quant software (Amersham Pharmacia), a reduction in signal intensity of more than 50% in one of the alleles in the tumor DNA compared with the paired normal DNA was defined as LOH. Many samples showed a reduction in signal intensity approaching 90%. Densitometry was undertaken on all samples where the reduction in signal intensity was difficult to quantify visually. Where sufficient material was available samples showing LOH were subjected to repeat amplification and analysis.

Comparison of clinical parameters The mean age at diagnosis and time from diagnosis were compared in those patients with and without LOH, and analyzed for statistical significance using unpaired t test, with Satterthwaite's correction for unequal variances. Standard errors were corrected for possible non-normality and unequal variances.

The number of treatments received prior to the sample skin biopsy was calculated for each patient. Treatments were divided into the following categories: psoralen + ultraviolet A, superficial low-dose radiotherapy, total skin electron beam therapy, interferon-α, extracorporeal photopheresis, topical chemotherapy, single agent oral chemotherapy, single agent intravenous chemotherapy, and multiagent intravenous chemotherapy. The number of treatments received by those with and without LOH were compared and tested for statistical significance using the above methods. Disease-specific death rates were calculated as the number of deaths from disease per year from diagnosis. Disease-specific death rates were similarly compared between these groups and also for different stages of disease and tested for statistical significance using the Kaplan–Meier nonparametric test.

In addition the effect of possible predictors of age at diagnosis, number of treatments received, stage of disease, and disease-specific death rates were estimated using an appropriate regression test: least-squares linear regression for age at diagnosis and number of treatments, logistic regression for cutaneous stage (T_1 and T_2 or T_3 and T_4) and Cox's proportional Hazards regression for time to death. In each case, estimates with confidence intervals were produced using robust standard errors to correct for possible mild non-normality. Predictors considered were advanced stage (T_3 or T_4), age and LOH (no LOH, LOH at one loci only, LOH at more than one loci). In each case, unadjusted (univariate) estimates were obtained prior to multivariate analysis.

RESULTS

Allelic loss in mycosis fungoides Examples of allelic loss are shown in **Fig 1**. The microsatellite markers analyzed on each arm and their approximate cytogenetic position is shown in **Table I**.

LOH was identified at one or more loci (range = 1–4) in 23 of 51 patients (45%), **Table II**. The number of informative patients for LOH studies and rates of loss on each of the 15 chromosomal arms analyzed are shown in **Table III**. The number of patients demonstrating LOH on each arm ranged from 0 to 9 (0–16%). The most frequent chromosomal arms showing allelic loss were 9p in 8 of 49 patients (16%), 10q in 6 of 50 patients (12%), 1p in 5 of 51 patients (10%) and 17p, also in 5 of 51 patients (10%).

The male to female ratio was similar in those with and without LOH; approximately 6 : 4. There was no significant difference in the mean age at diagnosis of mycosis fungoides in those with LOH at 56 y and those without at 51 y (p = 0.16, 95% C.I. = 49–58).

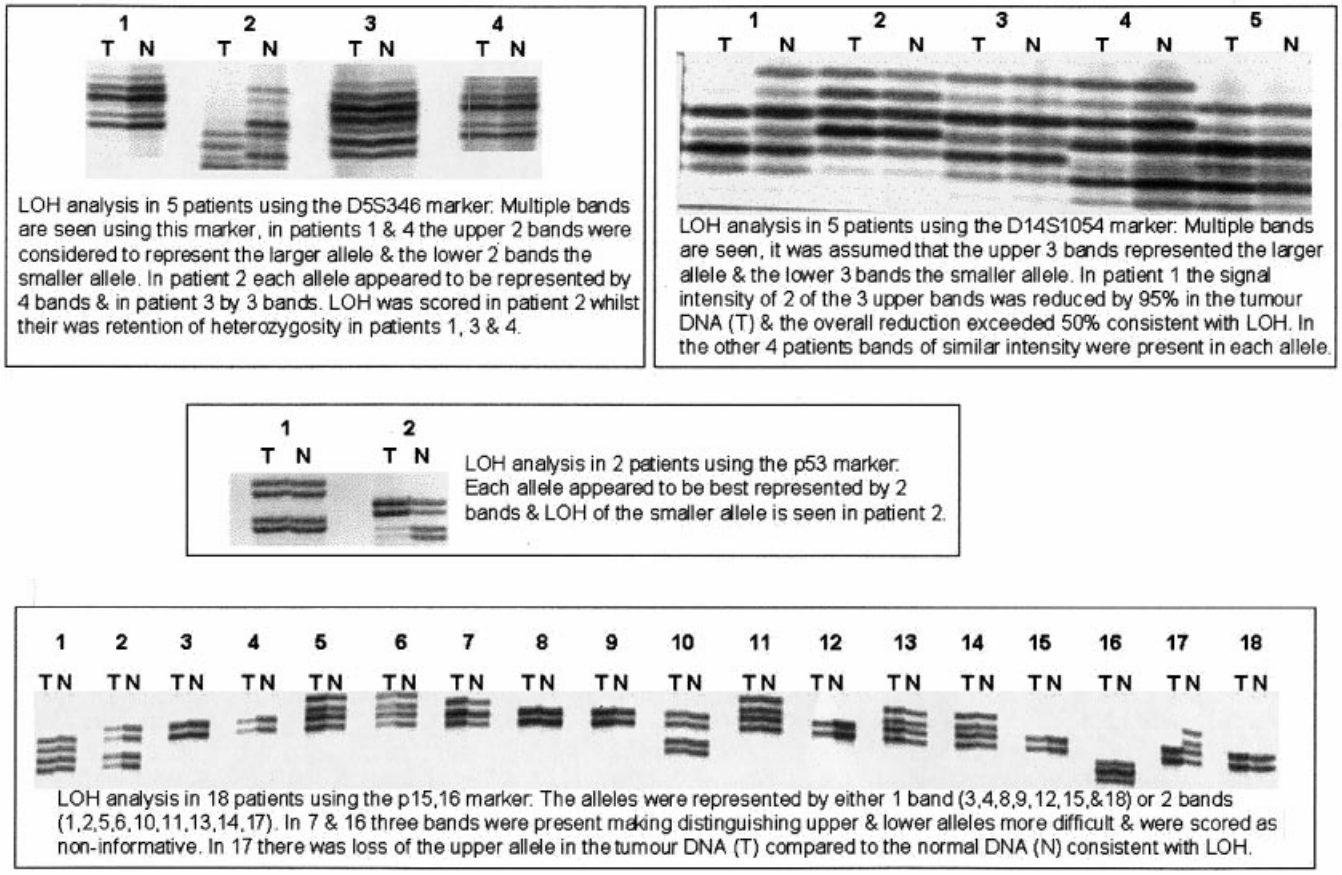

Figure 1. Microsatellite analysis using different markers produces different allelic banding patterns, LOH needs to be assessed in comparison with other samples using the same marker.

There was no significant difference in the mean number of treatments received by those with LOH at 1.3 and those without at 1.74 (p = 0.30, 95% C.I. = 1.2–2.0) **Table II**.

The mean follow-up from diagnosis was 5.1 y in those demonstrating LOH and 5.9 y in those without. Seven of 23 patients demonstrating LOH (30%) died from CTCL related disease during follow-up compared with 4 of 28 patients without LOH (14%). This equates to a disease-specific death rate of 0.06 per year in those with LOH, which is 3 times higher than those without LOH (p = 0.17), **Table II**. A graph showing Kaplan-Meier survival estimates in patients with and without LOH is shown in **Fig 2**.

A comparison of LOH rates with cutaneous stage of mycosis fungoides revealed that six of 14 patients with T_1 stage (43%), five of 16 patients with T_2 (31%), 10 of 17 with T_3 (59%), and two of four with T_4 stage disease (50%) demonstrated LOH at one or more loci. No patients with T_1 or T_4 stage disease died from CTCL-related causes during follow-up. Of the 16 patients with T_2 stage disease: one of five with LOH died from disease (20%) compared with one of 11 without (9%). This equates to a 2.5 times higher disease-specific death rate in those with early stage disease showing LOH than those of a similar stage without LOH. Of the 17 patients with T_3 stage disease: six of 10 with LOH died from disease (60%) compared with three of seven without (43%). This equates to disease-specific death rates of 0.10 and 0.08 per year, respectively.

Nodal disease was present in eight patients with mycosis fungoides (stage IVA) all with advanced cutaneous disease (T_3 or T_4), four of whom had LOH detected at one or more loci and four without LOH. One patient had visceral disease (stage IVB) had LOH detected on 17p only.

Comparing allelic loss on these chromosomal arms with cutaneous stage: five of 21 patients (24%) with advanced stage (T_3 or T_4) had LOH on 10q compared with only one of 30 (3%) with early stage disease (T_1 or T_2). Three of 21 patients (14%) with advanced stage had LOH on 17p compared with only two of 30 (7%) with early stage disease. On 9p four of 21 patients with advanced stage (19%) had LOH compared with four of 30 with early stage disease (13%) and on 1p LOH was found in two of 21 of patients with advanced stage (10%) and three of 30 with early stage disease (10%).

LOH in less than 10% of patients was detected on 2p, 3p, 5q, 6q, 11q, 13q, 14q, and 17q. No region of loss was detected on 12p, 19q, or 21q.

Table II. Clinical parameters in 51 patients with mycosis fungoides and comparison of these parameter in patients with and without loss of heterozygosity

	Cohort	No loss	LOH
Total no. of patients (%)	51 (100)	28 (55)	23 (45)
Mean age at diagnosis (y)	50	47	53
No. of treatments prior to biopsy	1.53	1.36	1.74
Mean follow-up from diagnosis (y)	5.5	5.9	5.1
No. died of disease (%)	11 (22)	4 (14)	7 (30)
Disease-specific death rate per year	0.04	0.02	0.06

Allelic loss in Sézary syndrome The microsatellite markers analyzed on each chromosomal arm and their approximate cytogenetic position are shown in **Table IV**. LOH was identified at one or more loci (range = 1–3) in 10 of 15 patients (67%). The number of informative patients for markers on each chromosomal arm varied between 11 and 15, mean 13. The

Table III. Loss of heterozygosity on each chromosomal arm in patients with mycosis fungoides

Chromosomal arm	No. of patients informative for LOH	No. of patients with LOH (%)
1p	51	5 (10)
2p	51	1 (2)
3p	49	2 (4)
5q	51	2 (4)
6q	51	1 (2)
9p	49	8 (16)
10q	50	6 (12)
11q	46	1 (2)
12p	51	0 (0)
13q	51	1 (2)
14q	51	2 (4)
17q	47	4 (9)
17p	51	5 (10)
19q	50	0 (0)
21q	45	0 (0)

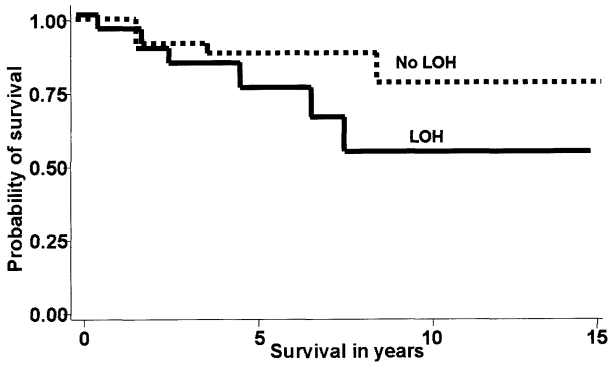

Figure 2. Graph demonstrating Kaplan–Meier survival estimates in patients with mycosis fungoides with and without LOH. $p = 0.17$.

number of patients demonstrating LOH on each arm is shown in **Table IV** and ranged from 0 to 6 (0–46%). High rates of allelic loss were identified on 9p and 17p: LOH on 9p was identified in six of 13 patients (46%) and on 17p in five of 12 patients (42%). Lower frequencies of loss were found on 10q, 2p, and 6q: LOH on 10q was detected in two of 14 patients (14%), on 2p in two of 14 patients (14%) and on 6q in one patient. LOH was not detected on 1p, 13q, or 17q.

The mean age at diagnosis of Sézary syndrome was 3 y younger in those patients demonstrating allelic loss, at 63 y, compared with those without LOH, at 60 y. The mean Sézary count was 77% in those with LOH compared with 59% in those without. In those samples demonstrating LOH 3 were from patients with stage III and seven with stage IVA disease, whereas the five samples that failed to show LOH were all from patients with stage III disease. The mean follow-up from diagnosis was 2.5 y in those demonstrating LOH and patients had received on average two different treatment modalities. In those without evidence of allelic loss the follow-up period was 5.6 y and patients had received on average three different treatments, **Table V**. All patients were alive on entry to the study. Three patients died during follow-up, two showing LOH and one without. Further analysis of survival data is not relevant due to small numbers and a short follow-up period.

DISCUSSION

This study has identified regions of allelic loss in both patients with mycosis fungoides and Sézary syndrome. Losses were identified in

Table IV. Microsatellite markers and their approximate chromosomal locations analyzed for loss of heterozygosity in patients with Sézary syndrome

Chromosomal region	Microsatellite markers	No. of patients informative for LOH	No. of patients with LOH (%)
1p32–36	D1S211, D1S201, D1S214	15	0 (0)
2p16–21	BAT26, D2S123	14	2 (14)
6q16.3–23.3	D6S283, D6S261	15	1 (7)
9p21	IFNA, p15,16	13	6 (46)
10q22–25	D10S541, D10S209, D10S215	14	2 (14)
13q14.2–22	L11910, D13S160	13	0 (0)
17q11.2–12	D17S250	11	0 (0)
17p13.1	BAT34C, p53	12	5 (42)

Table V. Clinical parameters in 15 patients with Sézary syndrome and comparison of these parameter in patients with and without loss of heterozygosity

	Cohort	No loss	LOH
Total no. of patients (%)	15 (100)	5 (33)	10 (67)
Mean age at diagnosis (y)	62	60	63
No. of treatments prior to study	2	3	2
Mean follow-up from diagnosis (y)	3.5	5.6	2.5
Mean Sézary count at entry to study	61	59	77

45% with mycosis fungoides including 37% with early and 57% with advanced cutaneous stages of disease and in 67% with Sézary syndrome. Allelic loss was detected on 80% of chromosomal arms studied but on most arms this involved low rates (< 5%) which may represent background genomic instability. The literature suggests that low-grade hematologic malignancies have a lower rate of LOH than high-grade malignancies and studies frequently cite rates of more than 10% as having probable significance (Takeuchi *et al*, 1995a; Cavé *et al*, 1996; Hatta *et al*, 1998; Mori *et al*, 1997). In our allelotyping study losses on 1p, 9p, 10q, and 17p were detected at rates over 10%. In particular these losses on 9p (46%) and 17p (42%) were more common in Sézary syndrome. These data suggest that the same putative tumor suppressor genes are important in both mycosis fungoides and Sézary syndrome malignancies and losses on these chromosomal regions are very unlikely to represent background genetic instability.

Allelic loss was consistently found at lower rates in mycosis fungoides and was more prevalent in advanced cutaneous disease. The higher rate of loss in advanced mycosis fungoides and Sézary syndrome may reflect the increased tumor burden in advanced disease enabling detection of LOH and tumor samples from patients with low tumor burden, as would be expected for patients with early disease, may be noninformative. In order to overcome this potential bias the purity of tumor DNA in all samples was assessed using TCR gene rearrangement studies and only those samples with a large dominant clonal T cell population were selected for this study. This technique, however is only semiquantitative and may have included some samples with a small clone that could have given rise to false negative LOH results, particularly in the early stages of disease where tumor burden is lower; however, the similar rates of LOH detected in all cutaneous stages of MF (43% with T_1, 31% with T_2, 59% with T_3 and 50% with T_4 stage disease) coupled with the high overall rate of LOH detected in this cohort (45%) suggests that false negative results are rare.

By only selecting samples with a large dominant clonal population patients with the earliest stages of mycosis fungoides were excluded. This may account for the high rates of loss detected in the early stages of disease, as only those with a high tumor burden were analyzed. LOH rates in patients with early cutaneous stages of mycosis fungoides and a small clone may be assessed by microdissecting tumor cells to enrich the tumor component. In addition most patients were selected for this study in whom either blood or lymph nodes did not show a T cell clone, which excluded many patients with widespread disease from the study, although this was in part overcome by extracting normal DNA from $CD8^+$ lymphocytes.

Chromosomal losses on 10q have been identified in previous cytogenetic studies, commonly involving 10q22–26 (Edelson *et al*, 1979; Limon *et al*, 1995; Karenko *et al*, 1997). A recent study using fluorescent *in situ* hybridization methods (FISH), capable of detecting chromosomal abnormalities in nondividing interphase cells and comparative genomic hybridization, a novel technique used to detect global losses or gains in tumor DNA compared with normal DNA, detected chromosomal loss on 10q in four patients with erythrodermic CTCL, with a minimal overlapping region of 10q25–26 (Karenko *et al*, 1999). Recent studies in other malignancies have identified chromosomal deletions involving 10q23–26, including transitional cell carcinoma (10q24.1–q24.2 and 10q26.1–26.2) (Cappellen *et al*, 1997) and endometrial carcinoma (10q22.1–23 and 10q25.2–26.3) (Peiffer *et al*, 1995). Deletion mapping in glioblastoma has also identified frequent loss at 10q23.3, which has subsequently been associated with deletions of the tumor suppressor gene PTEN (Wang *et al*, 1997). This study identified allelic loss on 10q in six of 50 patients (12%). Further detailed microsatellite analysis in these patients revealed a minimal overlapping region of deletion at 10q24 in 10 of 44 patients (23%), which in some cases was associated with homozygous deletion of the PTEN gene (Scarisbrick *et al*, 2000). These findings suggest that 10q22–26 harbors one or more tumor suppressor genes involved in the pathogenesis of various malignancies, including CTCL. Fine mapping the deletion on 10q using additional microsatellite markers increased the detection rate of LOH and the use of only one to three markers on each chromosomal arm in this allelotyping study may have underestimated the actual rates of allelic loss on individual arms (Hatta *et al*, 1999; Scarisbrick *et al*, 2000).

Chromosomal loss on 17p has been identified in CTCL (Limon *et al*, 1995; Thangavelu *et al*, 1997) and a study using fluorescent *in situ* hybridization analysis identified a specific region of deletion at 17p13 (Brito-Babpulle *et al*, 1997). We specifically selected markers close to the P53 tumor suppressor gene at 17p13.1 because overexpression has been found in high-grade CTCL (Lauritzen *et al*, 1995; McGregor *et al*, 1995) and several studies, including our own, have identified P53 gene mutations in a significant proportion of patients with advanced mycosis fungoides and Sézary syndrome (Marks *et al*, 1996; McGregor *et al*, 1999) suggesting that inactivation of the P53 gene is associated with disease progression in CTCL. Our LOH data support these findings: allelic loss on 17p was found in 10% of patients with mycosis fungoides, and was more prevalent in advanced cutaneous stages of disease (14%), and in 42% of patients with Sézary syndrome. Immunohistochemical studies of P53 gene expression in these patients found abnormal expression in the majority of atypical lymphocytes from the three cases of tumor stage disease suggesting the presence of biallelic P53 gene abnormalities and subsequent loss of gene function. In the two cases of early stage MF abnormal expression was only found in 5–10% of tumor cells suggesting the presence of a subclone with biallelic mutations (data not shown). Molecular studies looking for P53 gene mutations were not performed to confirm this. Intragenic mutation, however is an alternative mechanism of P53 gene inactivation and may be biallelic, thus the true rate of P53 gene inactivation in mycosis fungoides may be greater than suggested by LOH analysis.

Chromosomal rearrangements on 1p have frequently been detected in CTCL (Berger *et al*, 1988; Limon *et al*, 1995), and a common region of deletion has been identified on 1p22–36 (Thangavelu *et al*, 1997). We targeted this region and found LOH in 10% of patients with both early and advanced stage mycosis fungoides; however no loss on 1p was found in any of the 15 patients with Sézary syndrome. Allelic loss on 1p31–36 has been identified in a variety of solid tumors, including colon, breast, and ovary (Ragnarsson *et al*, 1999), as well as in chronic myeloid leukemia on 1p36 (Mori *et al*, 1998) and T cell non-Hodgkin's lymphoma on 1p32 (Perotti *et al*, 1999). Candidate tumor suppressor genes in this region include the P73 gene (1p36), which encodes a p53 homolog that may be inactivated in nodal lymphomas (Kawano *et al*, 1999) and the P18 gene (1p32) a cyclin-dependent kinase inhibitor, which is a homolog of the P15 and P16 genes. Absent P18 gene expression has been demonstrated in acute T cell leukemia cell lines (Hatta *et al*, 1997). In addition the TAL1 gene (1p32) encodes a hemopoietic transcription factor and deletions have been reported in T cell acute lymphoblastic leukemias (Janssen *et al*, 1993). Studies of these candidate tumor suppressor genes would be appropriate in determining their role in the pathogenesis of mycosis fungoides and particularly in disease initiation. In addition further LOH studies of patients with Sézary syndrome may be of value in determining if loss on 1p occurs in a subset of these patients.

Chromosomal abnormalities on 9p have previously been identified in CTCL (Van Vloten *et al*, 1980; Whang-Peng *et al*, 1982), including specific rearrangements involving 9p21–23 (Berger *et al*, 1988). The P15 and P16 genes are intricately linked on 9p21 and encode cyclin-dependent kinase inhibitors, which play a crucial part in the control of the cell cycle. Inactivation of the P15 and P16 genes is a frequent finding in high-grade non-Hodgkin's lymphoma (Gombart *et al*, 1995; Heyman *et al*, 1996), but has also been detected in a small study of patients with early cutaneous stages of mycosis fungoides (Peris *et al*, 1999). We selected markers that were close to the loci of the P15 and P16 genes for LOH analysis, including one that is located between the exons of these genes. We identified LOH on 9p21 in 46% of patients with Sézary syndrome and 16% with mycosis fungoides and allelic loss did not appear to be related to cutaneous stage. The P15 and P16 genes are frequently inactivated by hypermethylation of promoter sites, but rarely by small deletions or point mutations (Herman *et al*, 1997). Therefore, rates of LOH may underestimate the true prevalence of inactivation of these genes and further detailed studies of these genes would be beneficial

We did not detect any correlation between allelic loss and clinical features in mycosis fungoides: there was no significant difference in the age at diagnosis or number of treatments received by those with LOH and those without, which suggests that increasing age and multiple treatments do not predispose patients to allelic loss. Losses were more prevalent in advanced cutaneous stages of mycosis fungoides (T_3 and T_4) than early stage disease; however, lymph node involvement did not appear to be associated with an increased rate of LOH occurring in four patients with and four without LOH. In Sézary syndrome all patients have erythrodermic disease (T_4) but we found that those patients with LOH were more likely to have lymph node involvement (stage IVA) than those without LOH, all of whom had stage III disease.

In patients with mycosis fungoides the disease-specific death rate was found to be three times higher in those demonstrating LOH than those without, although this did not reach statistical significance ($p = 0.17$). It might appear that this finding can be explained by the increased prevalence of LOH in advanced stages of mycosis fungoides; however, the disease-specific death rate was 2.5 times higher in those with LOH and early stage disease, whereas in advanced cutaneous disease the disease-specific death rate was similar in those with or without LOH. This suggests that allelic loss may contribute to a more aggressive phenotype in mycosis fungoides particularly in early stage disease; however, patient numbers are small and further studies are required to validate this observation.

In Sézary syndrome survival data are difficult to interpret due to a shorter follow-up period; however the time from diagnosis was longer in those without LOH, at 5.6 y compared with those demonstrating LOH, at 2.5 y. In addition the five patients with LOH on more than one chromosomal arm had a follow up of only 1.8 y. This trend suggests that allelic loss may be more prevalent in those patients with Sézary syndrome who have a worse prognosis.

LOH studies have been extensively used to allelotype a wide variety of malignancies, but until now this technique has not been used in CTCL. This approach provides a sensitive method of screening chromosomes for regions of allelic loss and the likely sites of tumor suppressor genes involved in the pathogenesis of mycosis fungoides and Sézary syndrome; however this technique would not detect gross chromosomal abnormalities such as translocations, trisomy, or polyploidy, which have been frequently found in advanced CTCL (Whang-Peng *et al*, 1982; Berger *et al*, 1988; Thangavelu *et al*, 1997; Karenko *et al*, 1999). Furthermore, this method involves the comparison of tumor DNA with a large dominant clone and normal DNA, which may eliminate patients with the earliest stages of mycosis fungoides.

Allelic loss on 9p21 and 17p13 was present in almost half the patients with Sézary syndrome, suggesting that tumor suppressor genes at these loci, namely the P15, P16 (9p21), and P53 (17p13) genes may be frequently inactivated by LOH in Sézary syndrome. LOH on 10q and 17p was found in advanced stages of mycosis fungoides suggesting that tumor suppressor genes, such as the PTEN (10q23.3), Fas (10q24.1), and P53 (17p13.1) genes may be associated with disease progression in mycosis fungoides, whereas losses on 1p and 9p were not related to cutaneous stage suggesting that tumor suppressor genes in these regions, such as the P18 (1p32), TAL1 (1p32), and P16 (9p21) genes might be associated with early stage mycosis fungoides.

In eight patients with allelic loss DNA with a large dominant tumor population was available from both plaques and tumors for comparative LOH analysis: three patients with LOH on 9p21 and one with loss on 1p demonstrated allelic loss in DNA from both plaques and tumor lesions. Two patients with LOH on 10q, one with LOH on 17p, and one with LOH on 1p had losses confined to tumor samples with retention of heterozygosity in DNA from plaque stage disease. These data support the concept that a tumor suppressor gene(s) on 9p may be important in early disease, whereas those on 10q and 17p may play a part in disease progression; however patient numbers are too small to draw any further conclusions from these data and specific studies of different cutaneous stages of mycosis fungoides from individual patients, including microdissection of tumor cells from early stage disease will be required to substantiate this.

This study has identified several consistent regions of chromosomal loss involved in the pathogenesis of mycosis fungoides and Sézary syndrome. This provides the basis for further more detailed physical mapping studies and a rational approach for targeting analysis of specific tumor suppressor genes in CTCL.

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